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(54) Title: GENETIC SUPPRESSOR ELEMENTS AGAINST HUMAN IMMUNODEFICIENCY VIRUS

(57) Abstract

The present invention relates to genetic elements that suppress the activities of the human immunodeficiency virus (HIV). In particular, the invention relates to polynucleotides isolated from the HIV-1 genome, methods for isolating and identifying such polynucleotides, and methods for using them for the protection of human cells against HIV infection and/or replication.

GENETIC SUPPRESSOR ELEMENTS
AGAINST HUMAN IMMUNODEFICIENCY VIRUS

1. INTRODUCTION

5   The present invention relates to genetic elements that
suppress the activities of the human immunodeficiency virus
(HIV). In particular, the invention relates to
polynucleotides isolated from the HIV-1 genome, methods for
isolating and identifying such polynucleotides, and methods
10 for using them for the protection of human cells against HIV
infection and/or replication.

2. BACKGROUND OF THE INVENTION
2.1. THE HUMAN IMMUNODEFICIENCY VIRUS

15   The primary cause of acquired immunodeficiency syndrome
(AIDS) has been shown to be HIV (Barre-Sinoussi et al., 1983,
Science 220:868-870; Gallo et al., 1984, Science 224:500-
503). HIV causes immunodeficiency in an individual by
infecting important cell types of the immune system, which
20 results in their depletion. This, in turn, leads to
opportunistic infections, neurological dysfunctions,
neoplastic growth, and death.

        HIV is a member of the lentivirus family of retroviruses
(Teich et al., 1984, RNA Tumor Viruses, Weiss et al., eds.,
25 CSH-Press, pp. 949-956). Retroviruses are small enveloped
viruses that contain a diploid, single-stranded RNA genome,
and replicate via a DNA intermediate produced by a virally-
encoded reverse transcriptase, an RNA-dependent DNA
polymerase (Varmus, 1988, Science 240:1427-1439). There are
30 at least two distinct subtypes of HIV: HIV-1 (Barre-Sinoussi
et al., 1983, Science 220:868-870; Gallo et al., 1984,
Science 224:500-503) and HIV-2 (Clavel et al., 1986, Science
233:343-346; Guyader et al., 1987, Nature 326:662-669).
Genetic heterogeneity exists within each of these HIV
35 subtypes.

        CD4⁺ T cells are the major targets of HIV infection
because the CD4 cell surface protein acts as a cellular

receptor for HIV attachment (Dalgleish et al., 1984, Nature
312:763-767; Klatzmann et al., 1984, Nature 312:767-768;
Maddon et al., 1986, Cell 47:333-348). Viral entry into
cells is dependent upon viral protein gp120 binding to the
5 cellular CD4 receptor molecule (McDougal et al., 1986,
Science 231:382-385; Maddon et al., 1986, Cell 47:333-348).

2.2. HIV TREATMENT

HIV infection is pandemic and HIV-associated diseases
10 have become a world-wide health problem. Despite
considerable efforts in the design of anti-HIV modalities,
there is, thus far, no successful prophylactic or therapeutic
regimen against AIDS. However, several stages of the HIV
life cycle have been considered as potential targets for
15 therapeutic intervention (Mitsuya et al., 1991, FASEB J.
5:2369-2381). For example, virally-encoded reverse
transcriptase has been a major focus of drug development. A
number of reverse-transcriptase-targeted drugs, including
2',3'-dideoxynucleotide analogs such as AZT, ddI, ddC, and
20 ddT have been shown to be active against HIV (Mitsuya et al.,
1990, Science 249:1533-1544). While beneficial, these
nucleotide analogs are not curative, probably due to the
rapid appearance of drug resistant HIV mutants (Lander et
al., 1989, Science 243:1731-1734). In addition, the drugs
25 often exhibit toxic side effects, such as bone marrow
suppression, vomiting, and liver abnormalities.

Another stage of the HIV life cycle that has been
targeted is viral entry into the cells, the earliest stage of
HIV infection. This approach has primarily utilized
30 recombinant soluble CD4 protein to inhibit infection of CD4+ T
cells by some HIV-1 strains (Smith et al., 1987, Science
238:1704-1707). Certain primary HIV-1 isolates, however, are
relatively less sensitive to inhibition by recombinant CD4
(Daar et al., 1990, Proc. Natl. Acad. Sci. USA 87:6574-6579).
35 To date, recombinant soluble CD4 clinical trials have
produced inconclusive results (Schooley et al., 1990, Ann.
Int. Med. 112:247-253; Kahn et al., 1990, Ann. Int. Med.

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112:254-261; Yarchoan et al., 1989, Proc. Vth Int. Conf. on
AIDS, p. 564, MCP 137).

    The later stages of HIV replication, which involve
crucial virus-specific secondary processing of certain viral
5 proteins, have also been examined as possible anti-HIV drug.
targets. Late stage processing is dependent on the activity.
of a viral protease, and drugs have been developed to inhibit
this protease (Erickson, 1990, Science 249:527-533).
However, the clinical utility of these candidate drugs is
10 still in question.

    The lack of a satisfactory treatment for AIDS has led
investigators to gene therapy approaches. One form of gene
therapy involves the use of genetically-engineered viral
vectors to introduce toxic gene products to kill HIV-infected
15 cells. For instance, replication defective vectors have been
designed to introduce cell growth inhibitory genes into host
cells (WO 90/12087, October 18, 1980). One strategy
attempted by several groups involves the delivery of the
herpes simplex virus type 1 thymidine kinase (tk) toxin gene.
20 The tk gene product is toxic to mammalian cells only in the
presence of nucleoside analogs, such as ganciclovir
(Ventakash et al., 1990, Proc. Natl. Acad. Sci. USA 87: 8746-
8750; Brady et al., 1994, Proc. Natl. Acad. Sci. USA 91: 365-
369; WO 90/07936, July 26,1990). Diphtheria toxin gene has
25 also been used, and the gene was placed under the control of
cis-acting HIV regulatory sequences (U.S. Patent 5,306,631,
issued April 26, 1994). Others have utilized replication
incompetent mutants of HIV which have the potential to
express an inhibitory gene product in the presence of HIV tat
30 (WO 94/16060, July 21, 1994).

    Another form of gene therapy is designed to protect
virally-infected cells from cytolysis by specifically
disrupting viral replication. Efforts to identify
appropriate prot ctive genes have, in large part, been based
35 on an understanding of the molecular biology of HIV
replication. A few examples of this approach are as follows.

The HIV-1 *Rev* gene encodes a protein that is necessary for the expression of full length HIV-1 transcripts in infected cells and the production of HIV-1 virions. Transfection with one *Rev* mutant known as RevM10 has been

5 shown to protect the cells against HIV infection (Malim et al., 1992, J.Exp. Med. 176:1197; Bevec et al., 1992, Proc. Natl. Acad. Sci. USA 89:9870-74). Typically, the transfectants are resistant to HIV-1 infection for about 2 weeks from the time of inoculation before resistant variants

10 appear (Woffendin et al., 1994, Proc. Natl. Acad. Sci. USA 91: 11581-85).

In addition, *Rev* function can be interfered with by producing an excess of the binding site of the Rev protein, termed Rev Response Element (RRE), which prevents the binding

15 of Rev to RRE of viral transcripts. A "decoy" which consisted of a chimeric RNA composed of an RRE and a tRNA prevented infection of cultured cells for a period of greater than about 40 days (Lee et al., 1994, J.Virology 68:8254-64).

Alternatively, fusion proteins capable of binding to

20 viral env proteins have been made to prevent the production of HIV-1 virions. Examples include a fusion protein composed of CD4 and a lysosomal targeting protein procathepsin D, and an anti-env Fv which is secreted into the endoplasmic reticulum (Lin et al., WO 93/06216; Marasco et al., 1993,

25 Proc. Natl. Acad. Sci. USA 90:7889-93).

Antisense polynucleotides have also been designed to complex with and sequester the HIV-1 transcripts (Holmes et al., WO 93/11230; Lipps et al., WO 94/10302; Kretschmer et al., EP 594,881; and Chatterjee et al., 1992, Science

30 258:1485). Furthermore, an enzymatically active RNA, termed ribozyme, has been used to cleave viral transcripts. The ribozyme approach to forming an HIV-1 resistant hematopoietic cell line has been reported (Ojwang et al., 1992, Proc. Natl. Acad. Sci. USA 89:10802-06; Yamada et al., 1994, Gene Therapy

35 1:38-45; Ho et al., WO 94/26877; and Cech and Sullenger, WO 95/13379).

Roninson et al. described a method for isolating genetic
fragments from the HIV-1 genome capable of protecting a cell
from HIV-1 infection (U.S. Patent No. 5,217,889 and WO
92/07071). The method involves the preparation of an
5 expression library known as a Random Fragment Expression
(RFE) library that contains random sequence fragments of the
HIV-1 genome. Gene fragments referred to as HIV-1 Genetic
Suppressor Elements (HIV-1 GSE) are then selected from the
RFE library following an extensive selection procedure. The
10 selection step involves transfection of the RFE library into
a cell line to which HIV-1 infection is normally cytotoxic.
However, the low sensitivity of this selection step greatly
limits the practical use of the procedure. Moreover, no
specific GSE sequences were reported using this method that
15 were capable of suppressing HIV-1 infection.

3. **SUMMARY OF THE INVENTION**

The present invention relates to specific HIV-derived
polynucleotides herein referred to as GSE that suppress HIV
20 infection and/or replication in human cells, methods for
isolating and identifying such polynucleotides, and methods
for using them in the prevention and treatment of HIV
infection.

The invention is based, in part, on the Applicants'
25 discovery that nucleotide fragments can be isolated from the
HIV-1 genome, based on their ability to suppress the
activation of latent HIV-1 in a CD4· cell line. In this
connection, any cellular or viral marker associated with HIV
replication can be used to monitor the activation of latent
30 HIV. An example of such a marker is CD4, which is
conveniently monitored by using a specific antibody. While
the majority of the cells lose cell surface CD4 expression
after induction of the virus from latency, the cells
containing HIV-1 GSE retain CD4 expression. A number of
35 novel HIV-1 GSE polynucleotides are selected on the basis of
their ability to sustain CD4 expression by the induced cells,
and several of such sequences are further shown to protect

uninfected T cells from productive infection by HIV-1. The
GSE may function in the form of an RNA product or protein
product, both of which are within the scope of the invention.

    A wide range of uses are encompassed by the invention,
5 including but not limited to, AIDS treatment and prevention
by transferring GSE into HIV-1-susceptible cell types. For
example, GSE may be transferred into hematopoietic stem cells
in vitro followed by their engraftment in an autologous,
histocompatible or even histoincompatibile recipient. In an
10 alternative embodiment of the invention, any cells
susceptible to HIV infection may be directly transduced or
transfected with GSE *in vivo*.

4. BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1.   Schematic representation of the process of
using OM10.1 cells to select active HIV-1 GSE
clones from an HIV-1 RFE library.

Figure 2.   Percentage of CD4$^+$ OM10.1 cells diminishes
after TNF-α induction; TNF-induced cells, -■-;
20 uninduced cells,-◆-.

Figure 3.   HIV p24 level increases in OM10.1 cells after
TNF-α induction; TNF-induced cells,-◆-;
uninduced cells,-■-.

Figure 4.   GSE IGX-004 nucleotide sequence (SEQ ID NO:5)
25 in the sense orientation is selected for its
ability to maintain CD4 expression by OM10.1
cells after TNF-α induction.

Figure 5.   GSE IGX-024 nucleotide sequence (SEQ ID NO:6)
in the sense orientation is selected for its
30 ability to maintain CD4 expression by OM10.1
cells after TNF-α induction.

Figure 6.   GSE IGX-042 nucleotide sequence (SEQ ID NO:7)
in the sense orientation is selected for its
ability to maintain CD4 expression by OM10.1
35 cells after TNF-α induction.

Figure 7.   GSE IGX-009 nucleotide sequence (SEQ ID NO:8)
in the sense orientation is selected for its

ability to maintain CD4 expression by OM10.1
cells after TNF-α induction.

Figure 8.   GSE IGX-005 nucleotide sequence (SEQ ID NO:9)
in the sense orientation is selected for its
ability to maintain CD4 expression by OM10.1
cells after TNF-α induction.

Figure 9.   GSE IGX-230 nucleotide sequence (SEQ ID NO:10)
in the sense orientation is selected for its
ability to maintain CD4 expression by OM10.1
cells after TNF-α induction.

Figure 10.   GSE IGX-003 nucleotide sequence (SEQ ID NO:11)
in the anti-sense orientation is selected for
its ability to maintain CD4 expression by
OM10.1 cells after TNF-α induction.

Figure 11.   GSE IGX-170 nucleotide sequence (SEQ ID NO:12)
in the anti-sense orientation is selected for
its ability to maintain CD4 expression by
OM10.1 cells after TNF-α induction.

Figure 12.   Location of HIV GSE on the HIV-1 genome.
Arrows indicate antisense orientation
elements, while boxes indicate sense
orientation elements.

Figure 13.   Percentage of intracellular p24$^+$ cells after
infection with HIV-1$_{SF2}$ at a TCID$_{50}$ of 200.
CEM-ss cells (10^6) containing GSE were
harvested at 21, 28 and 35 days after
infection with HIV, stained with FITC-
conjugated anti-p24 antibody and analyzed by
flow cytometry. Transduced sequences: REVM10,
-◆-; plasmid DNA (negative control), -■-;
IGX-004, -▲-; IGX-230, -x-.

Figure 14.   Percentage of intracellular p24$^+$ cells after
infection with HIV-1$_{SF33}$ at a TCID$_{50}$ of 1000.
CEM-ss cells (10^6) containing GSE were
harvested at 9 days after infection with HIV,
stained with FITC-conjugated anti-p24 and
analyzed by flow cytometry.

Figure 15.   Percentage of CD4⁺ and p24⁻ OM10.1 cells containing IGX-230 constructs after TNF-α induction. OM10.1 cells containing 220 (plasmid DNA in LXSN vector), the IGX-230 sequence, or constructs representing the three potential reading frames of the IGX-230 sequence (A, B, C) were induced with TNF-α and analyzed 24 hours later for CD4 expression (dark column) and intracellular p24 (light column representing absence of p24) levels. A corresponds to *Tat*, B corresponds to *envelope*, and C corresponds to *Rev*.

Primers used for constructs representing the three potential reading frames (A, B, C) are as follows:

A. 5'-G <u>GAA TTC</u> <u>AAG CTT</u> GCC GCC ACC ATG GGC CCG ACG GAA TCG AA(g)
        EcoRI   HindIII   Met Gly Pro Thr Glu Ser Lys

B. 5'-G <u>GAA TTC</u> <u>AAG CTT</u> GCC GCC ACC ATG GAC GGG CCC GAC GGA ATC GAA
        EcoRI   HindIII   Met Asp Gly Pro Asp Gly Ile Glu

C. 5'-G <u>GAA TTC</u> <u>AAG CTT</u> GCC GCC ACC ATG GAC GGC TGG GCC CGA CGG AAT CGA
        EcoRI   HindIII   Met Asp Gly Trp Ala Arg Arg Asn Arg

Figure 16.   Percentage of intracellular p24⁺ CEM-ss cells containing the IGX-230 constructs (the three open reading frames are denoted as A, B, C) after infection with HIV-1$_{SF2}$ at a TCID$_{50}$ of 500. CEM-ss cells (10⁶) containing the IGX-230 constructs, 34 (plasmid DNA in LXSN), or the RevM10 in LNCX were harvested on the indicated days post infection, stained with FITC-conjugated anti-p24 and analyzed by flow cytometry. Negative control (34), -♦-; REVM10, -■-; A, -▲-; B, -x-; C, -*-.

Figure 17.   Percentage of intracellular p24⁺ CEM-ss cells containing the IGX-004 constructs (the two opening reading frames are denoted as 1 and 3)

after infection with HIV-1_{SF2} at a $TCID_{50}$ of
500. CEM-ss cells (10^6) containing the IGX-004
constructs or 34 (plasmid DNA in LXSN) were
harvested on the indicated days post

5   infection, stained with FITC-conjugated anti-
p24 and analyzed by flow cytometry. Construct
3 represents the *integrase* reading frame,
while construct 1 represents an alternative
reading frame. Negative control (34), -♦-;

10   construct 1, -■-; construct 3, -▲-.

Figure 18.   Percentage of intracellular p24⁺ CEM-ss cells
containing the IGX-009 sequence after
infection with HIV-1_{SF2} at a $TCID_{50}$ of 500.
CEM-ss cells (10^6) containing the IGX-009

15   construct or 34 (plasmid DNA in LXSN) were
harvested on the indicated days post
infection, stained with FITC-conjugated anti-
p24 and analyzed by flow cytometry. Negative
control (34), -♦-; IGX-009, -■-.

20

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to specific HIV-derived
polynucleotides identified by an improvement of the method
25 disclosed by Roninson et al. in United States Patent No.
5,217,889. More specifically, the improvement of the method
includes the use of a cell line containing a latent and
inducible HIV-1 provirus such as OM10.1. In addition, the
improvement also encompasses the use of a marker associated
30 with HIV infection such as CD4 to select for polynucleotides
from an HIV-1 RFE library that effectively suppress HIV-1
infection. The GSE selected by this procedure are also able
to protect uninfected cells from HIV infection.

Figure 1 presents a schematic drawing of one specific
35 method used to identify several GSE from a RFE library that
prevent the induction of latent HIV-1. The method includes
the steps of: 1) fragmenting the HIV-1 genom into 100-700

base pair (bp) fragments; 2) inserting the fragments into
expression vectors such that the fragments are transcribed
and translated to form an expression library; 3) transferring
the expression library into a population of cells containing
5 an inducible latent HIV-1 provirus or susceptible to HIV
infection; 4) selecting a subpopulation of cells which
contain a subset of the expression library enriched for HIV-1
GSE by monitoring the expression of a cellular or viral
marker associated with HIV infection; and 5) recovering the
10 GSE from the selected cell population. The method further
includes repetition of the aforementioned steps with a
secondary or tertiary library so that many rounds of
successive selection can be performed. The selection of GSE
can be performed by monitoring the continued expression of a
15 cellular marker such as CD4 or the decreased expression of a
viral marker such as p24 or gp120 using an antibody.

The invention is discussed in more detail in the
subsections below, solely for purposes of description and not
by way of limitation. For clarity of discussion, the
20 specific procedures and methods described herein are
exemplified using OM10.1 cells, CEM-ss cells, tumor necrosis
factor-alpha (TNF-α), an anti-CD4 antibody, and an anti-p24
antibody, but they are merely illustrative for the practice
of the invention. Analogous procedures and techniques are
25 equally applicable to isolating GSE from different subtypes
of HIV, utilizing any cell line containing an inducible
latent provirus or any cell line or freshly isolated cell
population susceptible to HIV infection, and any marker
associated with HIV infection that can be easily assayed.
30

5.1.   PREPARATION AND TRANSFECTION OF AN HIV-1 GSE LIBRARY

An HIV RFE library can be constructed from the DNA of a
plasmid or multiple plasmids that contain an HIV provirus
insert. HIV proviral DNA is first treated with enzymes to
35 produce randomly cleaved fragments. This can be conveniently
performed by DNase I cleavage in the presence of Mn⁺

(Roninson et al., Patent No. 5,217,889, column 5, lines 5-
20). Thereafter, the randomly cleaved genomic DNA are size
fractionated by gel electrophoresis. Fragments of between
100 and 700 bp are the preferred lengths for constructing RFE
5 libraries. Single strand breaks of the size-selected
fragments are repaired, e.g., by Klenow or T4 polymerase, and
ligated with 5' and 3' adaptors.

The 5' and 3' adaptors are selected to have non-cohesive
restriction sites so that each fragment can be inserted into
10 an expression vector in an oriented fashion. Further, the 5'
adaptor contains a start (ATG) codon to allow the translation
of the fragments which contain an open reading frame in the
correct phase.

After ligation with the adaptors the fragments are
15 inserted into appropriate expression vectors. Any expression
vector that results in efficient expression of the fragments
in host cells can be used. In a preferred embodiment a viral
based vector such as the retroviral vector LXSN is
exemplified (Miller and Rosman, 1989, BioTechniques 7:980).
20 Alternatively, adeno-associated virus vectors may also be
used for this purpose.

When viral-based vectors are used, the ligated vectors
are first transfected into a packaging cell line to produce
viral particles. For retroviral vectors, any amphotropic
25 packaging line such as PA317 (Miller and Buttimore, 1986,
Mol. Cell. Biol. 6:2895-2902; ATCC CRL #9078) may be used to
efficiently produce virus. In a preferred embodiment of the
invention, the viral vector also contains a selectable gene,
such as the neo^r gene, that allows selective growth of the
30 cells that contain the vector.

The number of independent clones present in each GSE
expression library may vary. In a preferred embodiment,
libraries of about 5×10^4 to 10^6 independent clones may be
used.

35

5.2. SELECTION OF GSE IN HIV-INFECTED CELLS

In a specific embodiment by way of example, OM10.1 cells
are used to select for GSE, and they are maintained in
conventional tissue culture as described in Butera (U.S.
5   Patent No. 5,256,534). The purpose of using OM10.1 cells for
the selection of HIV-1 GSE is that they contain a latent HIV-
1 provirus which is inducible by TNF-α. Other cell lines may
be similarly engineered with an inducible HIV provirus.
Examples of cell lines that are infected with latent HIV
10 include, but are not limited to U1, U33, 8E5, ACH-2, LL58,
THP/HIV and UHC4 (Bednarik and Folks, 1992, AIDS 6:3-16). A
variety of agents have been shown to be capable of inducing
latent HIV-infected cells, and these include TNF-α, TNF-β,
interleukins-1, -2, -3, -4 and -6, granulocyte-macrophage
15 colony stimulating factors, macrophage-colony stimulating
factors, interferon-γ, transforming growth factor-β, PMA,
retinoic acid and vitamin D3 (Poli and Fauci, 1992, AIDS Res.
Human Retroviruses 9:191-197).

The HIV-infected cells may be transduced with the HIV-1
20 RFE library by any technique well known in the art that is
appropriate to the vector system employed. In one embodiment
of the invention, the viral vector also contains a selectable
marker in addition to a random fragment of the HIV-1 genome.
A suitable marker is the neo^r gene, which permits selection by
25 the drug G-418. In alternative embodiments the multiplicity
of infection of the virions of the library is adjusted so
that pre-selection for cells that are transduced by the
vector is not needed.

In the case of OM10.1 cells, the transduced population
30 is treated with 10 U/ml TNF-α for a period of 24-72 hours and
preferably about 24 hours according to the method of Butera.
The activation of the latent HIV-1 provirus in OM10.1 can be
detected by the suppression of the cell surface CD4. It is
b lieved that viral protein gp120 binds to CD4 in the
35 cytoplasm, which prevents subsequent expression of CD4 on the
cell surface. Clones that are resistant HIV replication
continue to expr ss cell surface CD4. Such clones can be

selected by cell sorting using any conventional antibody
staining technique for CD4 and a fluorescence activated cell
sorter (FACS).

5   After selection for continued CD4 expression, the OM10.1
cells harboring putative GSE and sorted after TNF-α induction
are used to purify genomic DNA and the inserts amplified by
the polymerase chain reaction (PCR). Optionally, the
selected OM10.1 cells can be re-cultured under the selection
conditions for the marker gene, e.g., in G-418, to ensure
10   that the cells have retained the GSE derived from the HIV-1
RFE library.

The fraction of CD4˙ cells that have been transduced with
an HIV-1 RFE library can be compared with cells transduced
with an expression library consisting of the vector only. An
15   increased relative difference between the HIV-1 RFE library
and the control library can be found with each additional
round of TNF-α induction. Thus, in the preferred embodiment
of the invention there are at least two cycles of induction,
selection and reculturing before the HIV-1 GSE are recovered
20   from the cells for further characterization.

5.3.   **RECOVERY GSE FROM THE SELECTED CELLS**

After selection, specific GSE sequences can be recovered
from cells that continue to express CD4 following induction
25   of the latent HIV provirus by TNF-α. The recovery may be
performed by first expanding the population of selected cells
in culture and preparing their genomic DNA. The HIV-1-
associated GSE in this population are recovered by
amplification in PCR using the primers according to the
30   sequence of the linkers.

The recovered GSE can be introduced into an expression
vector as discussed in Section 5.1, *supra*. The resultant
HIV-1 GSE expression library is known as a secondary library.
The secondary library may utilize the same or a different
35   vector from that used for the construction of the primary
library. The secondary library may be transduced into

another cell population and the resultant population
selected, recultured and processed as described herein.

Additionally, each individually recovered element can be
inserted into cloning vectors for determining its specific
5 nucleotide sequence, its orientation and the portion of HIV
genome from which it is derived. Concurrently, the isolated
GSE can be analyzed to determine their minimal core sequences
and tested for their ability to protect previously uninfected
cells from HIV infection.
10   In addition to the sequences depicted in Figures 4-
11 (SEQ ID NOS:5-12), nucleotide sequences capable of
hybridizing to these sequences or their complementary
sequences under highly or less highly stringent hybridization
conditions are well within the scope of the invention.
15 Highly stringent hybridization conditions may be defined as
hybridization to filter-bound DNA in 0.5 M NaHPO4, 7% sodium
dodecyl sulfate (SDS), 1mM EDTA at 65°C, followed by washing
in 0.1 x SSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds,
1989, Current Protocols in Molecular Biology, Vol. I, Green
20 Publishing Associates, Inc., and John Wiley & Sons, Inc., New
York at p. 2.10.3). Less highly stringent conditions, such
as moderately stringent conditions, may be defined as
hybridizations carried out as described above, followed by
washing in 0.2x SSC/0.1% SDS at 42°C (Ausubel et al., 1989,
25 supra).

        5.4. **DETERMINATION OF CORE SEQUENCES OF GSE**
        The present invention also includes methods for
determining the core sequence of each GSE. This may be done
30 by comparing overlapping sequences of independently derived
GSE. Alternatively, GSE may be altered by additions,
substitutions or deletions and assayed for retention of HIV-
suppressive function. Alterations in the GSE sequences may
be generated using a variety of chemical and enzymatic
35 methods which are well known to those skilled in the art.
For example, oligonucleotide-directed mutagen sis may be
employed to alter the GSE sequence in a defined way and/or to

introduce restriction sites in specific regions within th
sequence. Additionally, deletion mutants may be generated
using DNA nucleases such as Bal 31 or Exo III and S1
nuclease. Progressively larger deletions in the GSE
5 sequences may be generated by incubating the DNA with
nucleases for increased periods of time (See Ausubel, et al.,
1989 Current Protocols for Molecular Biology, for a review of
mutagenesis techniques).

   The altered sequences may be evaluated for their ability
10 to suppress expression of HIV proteins such as p24 in
appropriate host cells. It is well within the scope of the
present invention that any altered GSE sequences that retain
their ability to suppress HIV infection may be incorporated
into recombinant expression vectors for further use.
15

   **5.5. PROTECTION OF UNINFECTED CELLS BY GSE AGAINST
        HIV-1 INFECTION**

   In order to confirm that the selected GSE can protect
uninfected cells from HIV-1 infection, the GSE may be
20 transferred into HIV susceptible host cells, followed by HIV
infection. Protection experiments can be performed in any
cell type that takes up the potential HIV-1 GSE and which is
otherwise susceptible to HIV infection. In a preferred
embodiment by way of example, the CEM-ss cell line is used
25 (Foley et al., 1965, Cancer 18:522-29). The use of CEM-ss
cells as targets for quantitative infectivity of HIV-1 has
been described by Nara & Fischinger (1988, Nature 322:469-
70). Other cell lines that are susceptible to HIV infection
include, but are not limited to, HUT-78, H9, Jurkat E6-1,
30 A3.01, U-937, AA-2, HeLa CD4⁺ and C8166.
   The test of the potential HIV-1 GSE can be performed
using the same expression vector system as that employed in
the RFE library transduction of cells during initial
selection steps. In other embodiments, the vector system can
35 be modified to achieve higher levels of expression, e.g., the
linkers can be employed to introduce a leader sequence that
increases the translational efficiency of the message. One

such sequence is disclosed by Kozak, 1994, Biochemie 76:815-
821.

Another way of testing the effectiveness of a GSE
against HIV is to determine how rapidly HIV-1 variants
5 develop that can negate the effects of the potential HIV-1
GSE. Such a test includes infection of a culture of
susceptible cells such as CEM-ss cells at a low multiplicity
of infection and repeatedly assaying the culture to determine
whether and how quickly HIV-1 infection becomes widespread.
10 The range of useful multiplicities of infection is between
about 100 to 1000 tissue culture infectious units ($TCID_{50}$) per
10^6 CEM-ss cells. The $TCID_{50}$ is determined by an endpoint
method and is important for determining the input
multiplicity of infection (moi).
15   A parameter that correlates with the development in the
test culture of HIV-1 strains that are resistant to the
effects of the potential HIV-1 GSE is the fraction of cells
that are infected in the culture. This fraction can be
determined by any means. Immunofluorescent staining with an
20 antibody specific for the HIV-1 p24 antigen of fixed
permeabilized cells is a convenient method for determining
the fraction of cells that is infected. Commercially
available reagents are suitable for performing such tests
(Lee et al., 1994, J. Virol. 68:8254-8264).
25   In Section 6.2, infra, three GSE were tested for their
ability to protect CEM-ss cells from infection with HIV-1
strains SF_2 and SF_{33}. Uninfected cells were transduced with
a LXSN construct containing either an irrelevant DNA or a GSE
sequence. Non-transduced cells were eliminated by exposure
30 to the selection agent, G-418. The percentage of p24· cells
was determined at specific time points post infection. The
results demonstrate that three out of three GSE tested are
able to protect a productive HIV-1 infection in susceptible
host cells.
35

5.6. USES OF GSE TO SUPPRESS HIV-1 INFECTION

Another aspect of the present invention is to use the isolated GSE against HIV infection prophylactically and therapeutically. In this connection, GSE operably linked to
5 a regulatory sequence such as a promoter that controls its expression may be transferred *in vitro* into any HIV-susceptible host cells or hematopoietic stem cells such as CD34⁺ cells obtained from bone marrow or mobilized peripheral blood, by any DNA transfer techniques well known in the art
10 such as electroporation, transfection or transduction, followed by transplantation of the cells into a recipient. When the GSE-containing cells differentiate *in vivo*, the progeny cells express the GSE and become resistant to HIV.

Alternatively, GSE may be directly administered *in vivo*
15 using a gene therapy expression vector. In particular, anti-HIV GSE can be delivered or transferred into CD4⁺ T cells in both HIV-infected or uninfected individuals to protect against development of HIV infection. GSE can also be transferred into stromal cells, including macrophages.
20   Expression vectors derived from viruses such as retroviruses, adeno-associated virus, herpes viruses, or bovine papilloma virus may be used for delivery of recombinant GSE into the targeted cell population. Methods which are well known to those skilled in the art can be used
25 to construct recombinant viral vectors containing a GSE sequence operably linked to a promoter that controls its expression (Sambrook *et al.*, 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel *et al.*, 1989, Current Protocols in Molecular Biology,
30 Greene Publishing Associates and Wiley Interscience, N.Y.). In a specific embodiment by way of example, GSE sequences were inserted into a retroviral vector. In cases where an adenovirus is used as an expression vector, a GSE sequence may be ligated to an adenovirus transcription-translation
35 control complex, <u>e.g.</u>, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination.

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Insertion in a non-essential region of the viral genome
(e.g., region E1 or E3) will result in a recombinant virus
that is viable and capable of expressing GSE in infected
hosts (Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA
5 81:3655-3659).

Alternatively, recombinant GSE nucleic acid molecules
can be reconstituted into liposomes for delivery to target
cells. Liposomes are spherical lipid bilayers with aqueous
interiors. All molecules that are present in an aqueous
10 solution at the time of liposome formation (in this case,
oligonucleotides) are incorporated into this aqueous
interior. The liposomal contents are both protected from the
external microenvironment and, because liposomes fuse with
cell membranes, are efficiently delivered into the cell
15 cytoplasm, obviating the need to neutralize the
polynucleotides' negative charge.

Specific initiation signals may also be required for
efficient translation of inserted GSE sequences. Exogenous
transcriptional control signals, including the ATG initiation
20 codon, must be provided. Furthermore, the initiation codon
must be in phase with the reading frame of the GSE sequence
to ensure translation of the entire insert. These exogenous
translational control signals and initiation codons can be of
a variety of origins, both natural and synthetic. The
25 efficiency of expression may be enhanced by the inclusion of
appropriate transcription enhancer elements, transcription
terminators, etc. (see Bittner et al., 1987, Methods in
Enzymol. 153: 516-544).

The isolated GSE sequences suppress HIV activity by
30 either encoding protein or RNA products. The present
invention encompasses any such protein product, including
fusion proteins, leader peptides and localization signals.
In addition, anti-sense RNA, DNA molecules and ribozymes that
function to inhibit HIV infection are also within the scope
35 of the invention. Anti-sense RNA and DNA molecules act to
directly block th translation of mRNA by binding to targeted

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mRNA and preventing protein translation. GSE may be
represented by structural RNAs which act as decoys.
        Some GSE may also form triplexes.
Oligodeoxyribonucleotides can form sequence-specific triple
5 helices by hydrogen bonding to specific complementary
sequences in duplexed DNA. Formation of specific triple
helices may selectively inhibit the replication and/or gene
expression of targeted genes by prohibiting the specific
binding of functional trans-acting factors.
10   Polynucleotides to be used in triplex helix formation
should be single stranded and composed of deoxynucleotides.
The base composition of these polynucleotides must be
designed to promote triple helix formation via Hoogsteen base
pairing rules, which generally require sizeable stretches of
15 either purines or pyrimidines to be present on one strand of
a duplex. Polynucleotide sequences may be pyrimidine-based,
which will result in TAT and CGC triplets across the three
associated strands of the resulting triple helix. The
pyrimidine-rich polynucleotides provide base complementarity
20 to a purine-rich region of a single strand of the duplex in a
parallel orientation to that strand. In addition,
polynucleotides may be chosen that are purine-rich, for
example, containing a stretch of G residues. These
polynucleotides will form a triple helix with a DNA duplex
25 that is rich in GC pairs, in which the majority of the purine
residues are located on a single strand of the targeted
duplex, resulting in GGC triplets across the three strands in
the triplex.
        Alternatively, the potential sequences that can be
30 targeted for triple helix formation may be increased by
creating a so called "switchback" oligonucleotide.
Switchback oligonucleotides are synthesized in an alternating
5'-3', 3'-5' manner, such that they base pair with first one
strand of a duplex and then the other, eliminating the
35 necessity for a sizeable stretch of either purines or
pyrimidines to be present on one strand of a duplex.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed

5 by endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of HIV RNA sequences. GSE represented by antisense RNA showing high affinity binding to target sequences can

10 also be used as ribozymes by addition of enzymatically active sequences known to those skilled in the art.

Both anti-sense RNA and DNA molecules, and ribozymes of the invention may be prepared by any method known in the art. These include techniques for chemically synthesizing

15 oligodeoxyribonucleotides well known in the art such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide

20 variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into host cells.

25 Various modifications to the nucleic acid molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3'

30 ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

Methods for introducing polynucleotides into cells or tissues include the insertion of naked polynucleotide, *i.e.*,

35 by injection into tissue, the introduction of a GSE in a cell *ex vivo*, *i.e.*, for use in autologous cell therapy, the use of a vector such as a virus, retrovirus, phage or plasmid, etc.

or techniques such as electroporation which may b used *in vivo* or *ex vivo*.

The GSE may be formulated and administered through a variety of means, including systemic, localized, or topical
5   administration. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. The mode of administration may be selected to maximize delivery to a desired target site in the body.
10   For systemic administration, route of injection includes, intramuscular, intravenous, intraperitoneal, and subcutaneous. The polynucleotides of interest are formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's
15 solution, or physiological saline buffer. In addition, the polynucleotides may be formulated in solid or lyophilized form, then redissolved or suspended immediately prior to use.

20 **6.   EXAMPLE: ISOLATION AND IDENTIFICATION
                    OF GSE AGAINST HIV-1**

        6.1. MATERIALS AND METHODS

              6.1.1. CONSTRUCTION OF RFE LIBRARY

        Cloned genomic DNA, plasmids pBENN6 (Cat. No. 343) and
25 pBENN7 (Cat. No. 342) or pBH10 (Cat. No. 90) from National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, which contained the entire HIV-1 genome was partially digested with DNAseI in the presence of manganese (Sambrook *et al.*, 1989, Molecular Cloning A Laboratory
30 Manual, Cold Spring Harbor Laboratory, N.Y.). Under these conditions, DNAseI is known to produce mostly double-stranded breaks. The resulting fragments were repaired with the Klenow fragment of DNA polymerase I and T4 polymerase and ligated to synthetic doubl -stranded adaptors.
35 The 5' adaptor (SEQ ID NOS:1 and 2):

                5'-CTCGGAATTCAAGCTTATGGATGGATG
                3'CCTTAAGTTCGAATACCTACCTAC-5'

The 3' adaptor (SEQ ID NOS:3 and 4):

               5'TGAGTGAGTGAATCGATGGATCCGTCT

          ACTCACTCACTTAGCTACCTAGGCAGATCCT-5'

       Thereafter, the mixture was digested with BamHI and

5 EcoRI, column purified and ligated to the retroviral vector
LXSN (Miller and Rosman, 1989, BioTechniques 7:980-990) cut
with EcoRI and BamHI. The ligation mixture was transformed
into *E. coli*. The total plasmid was purified from ~100,000
recombinant clones. The size distribution of the cloned

10 fragments was tested by PCR amplification using primers
derived from the vector sequences adjacent to the adaptors.

6.1.2.   CELL LINES AND REAGENTS

       The OM10.1 cells are available from the American Type

15 Culture Collection, Rockville, MD as CRL 10850 (Butera,
United States Patent No. 5,256,534). The CEM-ss cells are
available from the NIH AIDS Research and Reference Reagent
Program as Cat. No. 776. HIV-1$_{SF2}$ is available from NIH AIDS
Research and Reference Reagent Program as Cat. No. 275.

20   The anti-CD4 (Q4120PE) and anti-p24 (KC-57 FITC)
antibodies were purchased from Sigma and Coulter,
respectively. TNF-α was obtained from Boehringer Mannheim.
G418 was purchased from Gibco/BRL as Geneticin.

25   6.1.3.   TRANSDUCTION AND SELECTION OF GSE
       The plasmid DNA prepared according to the method of
Section 6.1.1. *supra*, was transfected into the packaging cell
line, PA317 (ATCC CRL #9078), and converted into retrovirus
for infection of OM10.1 cells. After G418 selection, the

30 OM10.1 cells harboring the entire RFE library were induced
with 10 U/ml of TNF-α at 37°C and, 24 hours later, were
stained with an antibody and sorted for CD4 expression. The
CD4⁺ cells were cultured, expanded in number, and genomic DNA
from the CD4⁺ cells was purified and used for PCR

35 amplification of inserts with the vector-derived primers.
The amplified mixture was digested with EcoRI and BamHI and

cloned back into the LXSN vector. The selection was
repeated.

6.1.4.   IMMUNOFLUORESCENCE AND FLOW CYTOMETRY

5   For the selection of CD4^{+} cells, 10^7 cells were washed
twice with Assay Buffer (500 ml PBS, 1 ml of 0.5 mM of EDTA
at pH 8, 0.5 ml of 10% sodium azide and 10 ml of fetal bovine
serum), and resuspended in 500 μl PBS to which 50 μl of anti-
CD4 antibody (Q4120 PE, Sigma) was added. After incubation
10 at 4°C for 30 min., 5 ml of Assay Buffer was added and the
cells centrifuged at 1200 rpm for 4 min. The cells were
washed twice with Assay Buffer before sorting by FACS. The
aforementioned procedure was performed under sterile
conditions.

15   In order to determine p24 expression in HIV-infected
cells, the cells were first washed twice with Assay Buffer.
About 10^6 cells were suspended in 100 μl Assay Buffer, mixed
with 2 ml of Ortho PermeaFix Solution (Ortho Diagnostics),
and incubated for 40 min. at room temperature. After
20 centrifugation at 1200 rpm for 4 min. at 4°C, the cells were
resuspended in 2 ml Wash Buffer (500 ml PBS, 25 ml fetal
bovine serum, 1.5% bovine serum albumin and 0.0055% EDTA) for
10 min. at room temperature. After centrifugation, the cells
were resuspended in 50 μl Wash Buffer and mixed with 1:500
25 dilution of an IgG$_{2a}$ antibody for 20 min. at 4°C, followed by
incubation with 5-10 μl of anti-p24 antibody (KC57-FITC,
Coulter) for 30 min. at 4°C. The cells were then washed
twice with Wash Buffer and analyzed by flow cytometry.

30   ### 6.1.5.   RECOVERY OF GSE AND SEQUENCE ANALYSIS
Genomic DNA was isolated from the selected population of
OM10.1 cells harboring putative GSE by resuspending the cell
pellet in 0.1% Triton X-100, 20μg/ml proteinase K in 1x PCR
buffer, incubating at 55°C for 1 hour, and boiling for 10
35 minutes. Genomic DNA was used for PCR amplification using
vector-derived primers, cloned into the LXSN vector, and
transformed into *E. coli* using techniques well known in the

art.   Individual plasmids were purified from *E. coli* clones
using QIAGEN plasmid kits.   Inserts were sequenced by the
dideoxy procedure (AutoRead Sequencing Kit, Pharmacia
Biotech) and run on a Pharmacia LKB A.L.F. DNA sequencer.
5 Sequences were analyzed using the DNASTAR program.

6.2.   RESULTS

HIV-1 GSE were isolated and identified according to the
general scheme of Figure 1.   An HIV-1 RFE library was
10 constructed from plasmids containing the entire genome of the
virus.   Following transfection of the entire library into a
packaging cell line, virus was transferred into OM10.1 cells
by co-cultivation.   The virally-transduced cells were
selected in culture medium containing G-418 to ensure the
15 retention of the viral vector.

When the transduced OM10.1 cells were treated with TNF-α
and stained with an antibody specific for the cell surface
molecule CD4, a rapid loss of CD4 expression was observed
(Figure 2).   In contrast, the vast majority of the uninduced
20 OM10.1 cells retained CD4 expression.   It is believed that
activation of the latent virus in OM10.1 cells by TNF-α led
to the production of viral protein gp120, which bound to
cytoplasmic CD4, thereby preventing its cell surface
translocation.   A diminution of CD4⁺ OM10.1 cells also
25 correlated with an increased production of viral protein p24
in the cells following TNF-α induction (Figure 3).

The small number of residual CD4⁺ cells were then stained
with an anti-CD4 antibody and sorted by FACS.   After the
cells were expanded in culture, the individual GSE
30 polynucleotides were recovered by PCR amplification and their
nucleotide sequences determined.   Figures 4-9 present the
nucleotide sequences of six polynucleotides (IGX-004, IGX-
024, IGX-042, IGX-009, IGX-005, IGX-230) corresponding to GSE
in the sense orientation, whereas Figures 10 and 11 present
35 the nucleotide sequences of two GSE (IGX-003 and IGX-170) in
the antisense orientation.   Figure 12 shows the location of
the isolated GSE on the HIV-1 g nome.   These GSE are

distributed throughout different regions of the genome. For
example, the IGX-004 GSE (SEQ ID NO:5) is located within the
integrase gene, the IGX-009 GSE (SEQ ID NO:8) is located
within the *Nef* gene, and the IGX-230 GSE (SEQ ID NO:10)
5 overlaps the *Rev* and *Tat* genes.

Three GSE, IGX-230, IGX-004 and IGX-009, were further
tested for their ability to protect uninfected human T cells
from a productive HIV-1 infection. The plasmids containing
each of these sequences were transduced into CEM-ss cells
10 followed by G418 selection. The Rev transdominant mutant,
RevM10 (Malin et al., 1989, Cell 58:205), and a LXSN vector
containing an irrelevant piece of plasmid DNA (34) were used
as controls. The G418 resistant cells were 99% CD4$^+$, and were
then infected with low titers (TCID$_{50}$ of 200) of HIV-1$_{SF2}$ and
15 high titers (TCID$_{50}$ of 1000) HIV-1$_{SF33}$. The cells were removed
at 21, 28 and 35 days after infection with the low titers and
at 9 days after infection with the high titers, and stained
with a fluorescinated-anti-p24 antibody as an indicator of
HIV infection.

20   Figure 13 shows that the IGX-004 sequence was able to
suppress infection with HIV-1$_{SF2}$, as evidenced by the low
percentage of p24$^+$ cells over one month after infection. Only
1%, 3% and 44% of the cells transduced with the IGX-004
sequence were positive for p24 expression on days 21, 28 and
25 35, respectively. For negative control (34), 64% of the
cells were positive for p24 at day 21, and 99% of the cells
became p24$^+$ cells by day 28. Transduction of RevM10 led to 8%
p24$^+$ cells on day 21, 87% p24$^+$ cells on day 28 and 95% p24$^+$
cells on day 35. A pattern similar to RevM10 was seen for
30 the IGX-230 sequence with 4% of p24$^+$ cells on day 21, 80% on
day 28 and 92% on day 35. Thus, the IGX-230 sequence
produced an intermediate suppressive effect between that of
negative control and IGX-004 sequences.

Additionally, the percentage of intracellular p24$^+$ cells
35 was also determined at 9 days after infection of CEM-ss cells
with high titers of HIV-1$_{SF33}$ (Figure 14). Again, the IGX-004
sequence was most effective at suppressing p24 expr ssion at

- 25 -

29%, whereas the negative control (34) and the IGX-230
sequences produced 97% and 45% p24⁺ cells, respectively.

Figure 12 shows that the GSE IGX-230 encompasses
portions of both the *Rev* and *Tat* genes. In order to
5 determine the functional reading frame of IGX-230, constructs
representing all three potential reading frames were made and
transferred into OM10.1 cells. The constructs contained
sequences in three open reading frames using 5' adaptors that
contained the Kozak sequence (Kozak, 1994, Biochemie 76:815-
10 821). After TNF-α induction, the cells were analyzed 24
hours later for CD4 and p24 expression. Interestingly, all
three open reading frames of IGX-230 suppressed latent HIV
activation, as measured by the continued expression of CD4
(Figure 15). CD4 expression directly correlated with a
15 decrease of p24 levels in the cells. Furthermore, when the
constructs containing the three open reading frames were
transferred into CEM-ss cells following infection with HIV-
1$_{SF2}$, they were also able to suppress the expression of p24
over time (Figure 16). These results suggest that IGX-230
20 may function as a structural RNA rather than as a coding
sequence for a protein product.

The same experiment was performed with two constructs of
the GSE IGX-004 which mapped within the HIV-1 *integrase* gene.
Figure 17 demonstrates that the construct corresponding to
25 the *integrase* reading frame strongly suppressed the levels of
p24 in CEM-ss cells after HIV-1 infection for over three
weeks. In contrast, the construct representing an
alternative reading frame was not active.

A third GSE, IGX-009, which mapped within the *Nef* gene
30 was also able to sustain CD4 expression and suppress p24
levels in OM10.1 cells after TNF-α induction. In addition,
the IGX-009 sequence protected CEM-ss cells from HIV-1$_{SF2}$
infection, as shown by its ability to suppress intracellular
p24 levels as compared to a control sequence (Figure 18).

35   In conclusion, a large number of GSE have been isolated
from the HIV-1 genome based on their ability to maintain CD4
expression in OM10.1 cells after activation of latent HIV by

induction with TNF-α. The isolated GSE contain nucleotide
sequences in both sense and anti-sense orientations, and are
mapped to different regions of the HIV-1 genome. Several
elements corresponding to portions of the *integrase*, *Nef* and
5 *Rev/Tat* genes are able to suppress HIV-1 infection of T cells
by reducing p24 levels in infected cells. Such
polynucleotides are useful in protecting the infection by
and/or suppressing the replication of HIV-1 in human host
cells.

10   The present invention is not to be limited in scope by
the exemplified embodiments, which are intended as
illustrations of individual aspects of the invention.
Indeed, various modifications for the invention in addition
to those shown and described herein will become apparent to
15 those skilled in the art from the foregoing description and
accompanying drawings. Such modifications are intended to
fall within the scope of the appended claims.

     All publications cited herein are incorporated by
reference in their entirety.
20

25

30

35

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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   (ii) TITLE OF INVENTION: GENETIC SUPPRESSOR ELEMENTS AGAINST
        HUMAN IMMUNODEFICIENCY VIRUS

  (iii) NUMBER OF SEQUENCES: 12

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    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: unknown

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCGGAATTC AAGCTTATGG ATGGATG   27

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 24 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: unknown

       (ii) MOLECULE TYPE: cDNA

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTTAAGTTC GAATACCTAC CTAC   24

(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 27 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: unknown

       (ii) MOLECULE TYPE: cDNA

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGAGTGAGTG AATCGATGGA TCCGTCT   27

(2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 31 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: unknown

       (ii) MOLECULE TYPE: cDNA

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACTCACTCAC TTAGCTACCT AGGCAGATCC T   31

(2) INFORMATION FOR SEQ ID NO:5:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 172 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: unknown

       (ii) MOLECULE TYPE: cDNA

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ACATTTAGAA GGAAAAGTTA TCCTGGTAGC AGTTCATGTA GCCAGTGGAT ATATAGAAGC   60

AGAAGTTATT CCAGCAGAAA CAGGGCAGGA AACAGCATAC TTTCTTTTAA AATTAGCAGG   120

AAGATGGCCA GTAAAAACAA TACATACAGA CAATGGCAGC AATTTCACCA GT   172

(2) INFORMATION FOR SEQ ID NO:6:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 194 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: unknown

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACAAATAGGA TGGATGACAA ATAATCCACC TATCCCAGTA GGAGAAATTT ATAAAAGATG   60

GATAATCCTG GGATTAAATA AAATAGTAAG AATGTATAGC CCTACCAGCA TTCTGGACAT   120

AAGACAAGGA CCAAAAGAAC CCTTTAGAGA CTATGTAGAC CGGTTCTATA AAACCTTGTT   180

GGTCCAAAAT GCGA   194

(2) INFORMATION FOR SEQ ID NO:7:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 231 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: unknown

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATGACAAAT AATCCACCTA TCCCAGTAGG AGAAATTTAT AAAAGATGGA TAATCCTGGG   60

ATTAAATAAA ATAGTAAGAA TGTATAGCCC TACCAGCATT CTGGACATAA GACAAGGACC   120

AAAAGAACCC TTTAGAGACT ATGTAGACCG GTTCTATAAA ACTCTAAGAG CCGAGCAAGC   180

TTCACAGGAG GTAAAAAATT GGATGACAGA AACCTTGTTG GTCCAAAATG C   231

(2) INFORMATION FOR SEQ ID NO:8:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 192 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: unknown

        (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTGGGAGCAG TATCTCGAGA CCTGGAAAAA CATGGAGCAA TCACAAGTAG CAATACAGCA   60

GCTACTAATG CTGATTGTGC CTGGCTAGAA GCA   93

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 110 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: unknown
        (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCTCAGACCC TTTTAGTCAG TGTGGAAAAT CTCTAGCAGT GGCGCCCGAA CAGGGACTTG   60

AAAGCGAAAG GGAAACCAGA GGAGCTCTCT CGACGCAGGA CTCGGCTTGC   110

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 126 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GACGGCTGGG CCCGACGGAA TCGAAGAAGA AGGTGGAGAG AGAGACAGAG ACAGATCCGT   60

TCGATTAGTG TATGGATTCT TAGCACTTAT CTGGGAAGAT CTGCGGAGCC TGTGCCTCTT   120

CAGCTACCGC CGCT   134

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 338 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCAAATATTG GTGGAATCTC CTACAGTATT GGAGTCAGGA ACTAAAGAAT AGTGCTGTTA   60

GCTTGCTCAA TGCCACAGCC ATAGCAGTAG CTGAGGGGAC AGATAGGGTT ATAGAAGTAG   120

TACAAGGAGC TTGTAAGCTA TTCGCCACAT ACCTAGAAGA ATAAGACAGG GCTTGGAAAG   180

GATTTTGCTA TAAGATGGGT GGCAAGTGGT CAAAAAGTAG TGTGGTTGGA TGGCCTACTG   240

TAAGGGAAAG AATGAGACGA GCTGAGCCAG CAGCAGATGG GGTGGGAGCA GCATCTCGAG   300

ACCTGGAAAA ACATGGAGCA ATCACAAGTA GCAATACA   338

(2) INFORMATION FOR SEQ ID NO:12:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 187 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: unknown

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGCACACAAA GGAATTGGCG GAAATGAACA AGTAGATAAA TTAGTCAGTG CTGGAATCAG   60

GAAAGTACTA TTTTTAGATG GAATAGATAA GGCCCAAGAT GAACATGAGA AATATCACAG   120

TAATTGGAGA GCAATGGCTA GTGATTTTAA CCTGCCACCT GTAGTAGCAA AAGAAATAGT   180

AGCCAGC   187

WHAT IS CLAIMED IS:

1. An isolated polynucleotide, comprising a nucleotide sequence derived from an HIV-1 genome operably linked to a promoter, and expression of said sequence in a host cell suppresses infection by HIV-1.

2. The polynucleotide of claim 1, in which suppression of infection by HIV-1 is measured by continued CD4 expression in the cell following HIV-1 infection.

3. The polynucleotide of claim 1, in which suppression of infection by HIV-1 is measured by decreased viral p24 expression in the cell following HIV-1 infection.

4. The polynucleotide of claim 1, in which the sequence is depicted in SEQ ID NO:5 or which is capable of selectively hybridizing to the sequence of SEQ ID NO:5 or its complement.

5. The polynucleotide of claim 1, in which the sequence is depicted in SEQ ID NO:6 or which is capable of selectively hybridizing to the sequence of SEQ ID NO:6 or its complement.

6. The polynucleotide of claim 1, in which the sequence is depicted in SEQ ID NO:7 or which is capable of selectively hybridizing to the sequence of SEQ ID NO:7 or its complement.

7. The polynucleotide of claim 1, in which the sequence is depicted in SEQ ID NO:8 or which is capable of selectively hybridizing to the sequence of SEQ ID NO:8 or its complement.

8. The polynucleotide of claim 1, in which the sequence is depicted in SEQ ID NO:9 or which is capable of

selectively hybridizing to the sequence of SEQ ID NO:9 or its
complement.

9.   The polynucleotide of claim 1, in which the
5 sequence is depicted in SEQ ID NO:10 or which is capable of
selectively hybridizing to the sequence of SEQ ID NO:10 or
its complement.

10.   The polynucleotide of claim 1, in which the
10 sequence is depicted in SEQ ID NO:11 or which is capable of
selectively hybridizing to the sequence of SEQ ID NO:11 or
its complement.

11.   The polynucleotide of claim 1, in which the
15 sequence is depicted in SEQ ID NO:12 or which is capable of
selectively hybridizing to the sequence of SEQ ID NO:12 or
its complement.

12.   An expression vector comprising a nucleotide
20 sequence derived from an HIV-1 genome operably linked to a
promoter, and expression of said sequence in a host cell
suppresses infection by HIV-1.

13.   The expression vector of claim 12 in which the
25 nucleotide sequence is capable of hybridizing under stringent
conditions but for the degeneracy of the genetic code to the
sequence of SEQ ID NO:5 or its complement.

14.   The expression vector of claim 12 in which the
30 nucleotide sequence is capable of hybridizing under stringent
conditions but for the degeneracy of the genetic code to the
sequence of SEQ ID NO:6 or its complement.

15.   The expression vector of claim 12 in which the
35 nucleotide sequence is capable of hybridizing under stringent
conditions but for the d generacy of the genetic code to the
sequence of SEQ ID NO:7 or its complement.

16. The expression vector of claim 12 in which the nucleotide sequence is capable of hybridizing under stringent conditions but for the degeneracy of the genetic code to the sequence of SEQ ID NO:8 or its complement.

5

17. The expression vector of claim 12 in which the nucleotide sequence is capable of hybridizing under stringent conditions but for the degeneracy of the genetic code to the sequence of SEQ ID NO:9 or its complement.

10

18. The expression vector of claim 12 in which the nucleotide sequence is capable of hybridizing under stringent conditions but for the degeneracy of the genetic code to the sequence of SEQ ID NO:10 or its complement.

15

19. The expression vector of claim 12 in which the nucleotide sequence is capable of hybridizing under stringent conditions but for the degeneracy of the genetic code to the sequence of SEQ ID NO:11 or its complement.

20

20. The expression vector of claim 12 in which the nucleotide sequence is capable of hybridizing under stringent conditions but for the degeneracy of the genetic code to the sequence of SEQ ID NO:12 or its complement.

25

21. A genetically engineered host cell that contains the recombinant vector of claim 12, 13, 14, 15, 16, 17, 18, 19 or 20.

30   22. The genetically engineered host cell of claim 21 which expresses the nucleotide sequence as a peptide.

23. The genetically engineered host cell of claim 21 which expresses the nucleotide sequence as a RNA.

35

24. A method for identifying a genetic suppressor
element that suppresses infection of host cells by HIV-1,
comprising:

       (a)   constructing an expression library of
5   nucleotide sequences from an HIV-1 genome;

       (b)   transferring the expression library into host
              cells that contain an inducible latent HIV-1
              provirus;

       (c)   activating the latent provirus with an
10   inducing agent; and

       (d)   selecting the cells that contain a nucleotide
              sequence which suppresses HIV-1 infection by
              measuring levels of a detectable marker in the
              cells.

15

25. The method of claim 24, in which the host cells are
OM10.1.

26. The method of claim 24, in which the inducing agent
20 is TNF-α.

27. The method of claim 24, in which the marker is
cellular CD4.

25   28. The method of claim 27, in which expression of the
marker is retained by the host cells.

29. The method of claim 24 in which the marker is viral
protein p24.
30

30. The method of claim 24 in which the marker is viral
protein gp120.

31. The method of claim 29 or 30 in which expression of
35 the marker is suppressed in the host cells.

32.   A method for protecting a host cell from HIV-1
infection, comprising introducing an effective amount of a
polynucleotide derived from an HIV-1 genome, operably linked
to a promoter into the host cell, and suppressing HIV-1
5 infection in the cell by expressing said sequence.

33.   The method of claim 32, in which the polynucleotide
is introduced into the host cell *in vitro*.

10   34.   The method of claim 32, in which the polynucleotide
is introduced into the host cell *in vivo*.

35.   The method of claim 32, in which the polynucleotide
comprises a nucleotide sequence as depicted in SEQ ID NO:5 or
15 which is capable of selectively hybridizing to the sequence
of SEQ ID NO:5 or its complement.

36.   The method of claim 32, in which the polynucleotide
comprises a nucleotide sequence as depicted in SEQ ID NO:6 or
20 which is capable of selectively hybridizing to the sequence
of SEQ ID NO:6 or its complement.

37.   The method of claim 32, in which the polynucleotide
comprises a nucleotide sequence as depicted in SEQ ID NO:7 or
25 which is capable of selectively hybridizing to the sequence
of SEQ ID NO:7 or its complement.

38.   The method of claim 32, in which the polynucleotide
comprises a nucleotide sequence as depicted in SEQ ID NO:8 or
30 which is capable of selectively hybridizing to the sequence
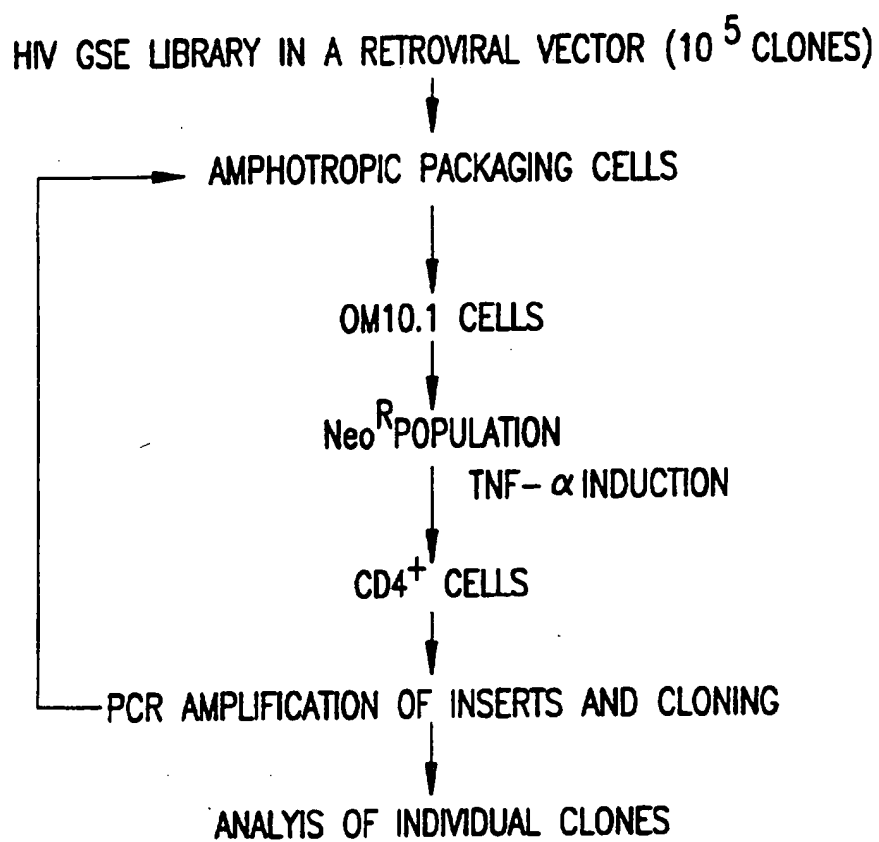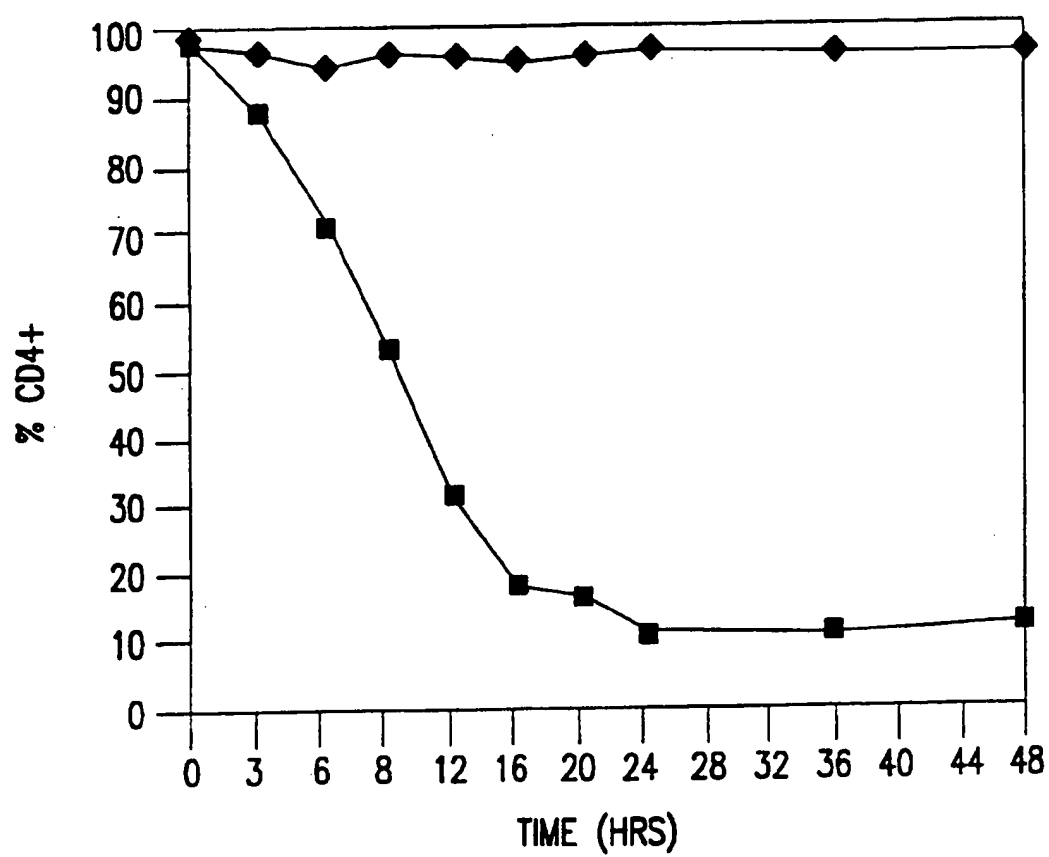of SEQ ID NO:8 or its complement.

39.   The method of claim 32, in which the polynucleotide
comprises a nucleotide sequence as depicted in SEQ ID NO:9 or
35 which is capable of selectively hybridizing to the sequence
of SEQ ID NO:9 or its complement.

40. The method of claim 32, in which the polynucleotide comprises a nucleotide sequence as depicted in SEQ ID NO:10 or which is capable of selectively hybridizing to the sequence of SEQ ID NO:10 or its complement.

5

41. The method of claim 32, in which the polynucleotide comprises a nucleotide sequence as depicted in SEQ ID NO:11 or which is capable of selectively hybridizing to the sequence of SEQ ID NO:11 or its complement.

10

42. The method of claim 32, in which the polynucleotide comprises a nucleotide sequence as depicted in SEQ ID NO:12 or which is capable of selectively hybridizing to the sequence of SEQ ID NO:12 or its complement.

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1/12

HIV GSE LIBRARY IN A RETROVIRAL VECTOR (10^5 CLONES)

AMPHOTROPIC PACKAGING CELLS

OM10.1 CELLS

Neo^R POPULATION

TNF-α INDUCTION

$CD4^+$ CELLS

PCR AMPLIFICATION OF INSERTS AND CLONING

ANALYIS OF INDIVIDUAL CLONES

FIG.1

FIG.2

FIG.3

ACATTTAGAA GGAAAAGTTA TCCTGGTAGC AGTTCATGTA GCCAGTGGAT
ATATAGAAGC AGAAGTTATT CCAGCAGAAA CAGGGCAGGA AACAGCATAC
TTTCTTTTTAA AATTAGCAGG AAGATGGCCA GTAAAAACAA TACATACAGA
CAATGGCAGC AATTTCACCA GT

FIG.4

ACAAATAGGA TGGATGACAA ATAATCCACC TATCCCAGTA GGAGAAATTT
ATAAAAGATG GATAATCCTG GGATTAAATA AAATAGTAAG AATGTATAGC
CCTACCAGCA TTCTGGACAT AAGACAAGGA CCAAAAGAAC CCTTTAGAGA
CTATGTAGAC CGGTTCTATA AAACTCTAAG AGCCGAGCAA GCTTCACAGG
AGGTAAAAAA TTGGATGACA GAAACCTTGT TGGTCCAAAA TGCGA

FIG.5

GATGACAAAT AATCCACCTA TCCCAGTAGG AGAAATTTAT AAAAGATGGA
TAATCCTGGG ATTAAATAAA ATAGTAAGAA TGTATAGCCC TACCAGCATT
CTGGACATAA GACAAGGACC AAAAGAACCC TTTAGAGACT ATGTAGACCG
GTTCTATAAA ACTCTAAGAG CCGAGCAAGC TTCACAGGAG GTAAAAAATT
GGATGACAGA AACCTTGTTG GTCCAAAATG C

FIG.6

GTGGGAGCAG TATCTCGAGA CCTGGAAAAA CATGGAGCAA TCACAAGTAG
CAATACAGCA GCTACTAATG CTGATTGTGC CTGGCTAGAA GCA

FIG.7

CCTCAGACCC TTTTAGTCAG TGTGGAAAAT CTCTAGCAGT GGCGCCCGAA
CAGGGACTTG AAAGCGAAAG GGAAACCAGA GGAGCTCTCT CGACGCAGGA
CTCGGCTTGC

FIG.8

GACGGCTGGG CCCGACGGAA TCGAAGAAGA AGGTGGAGAG AGAGACAGAG
ACAGATCCGT TCGATTAGTG TATGGATTCT TAGCACTTAT CTGGGAAGAT
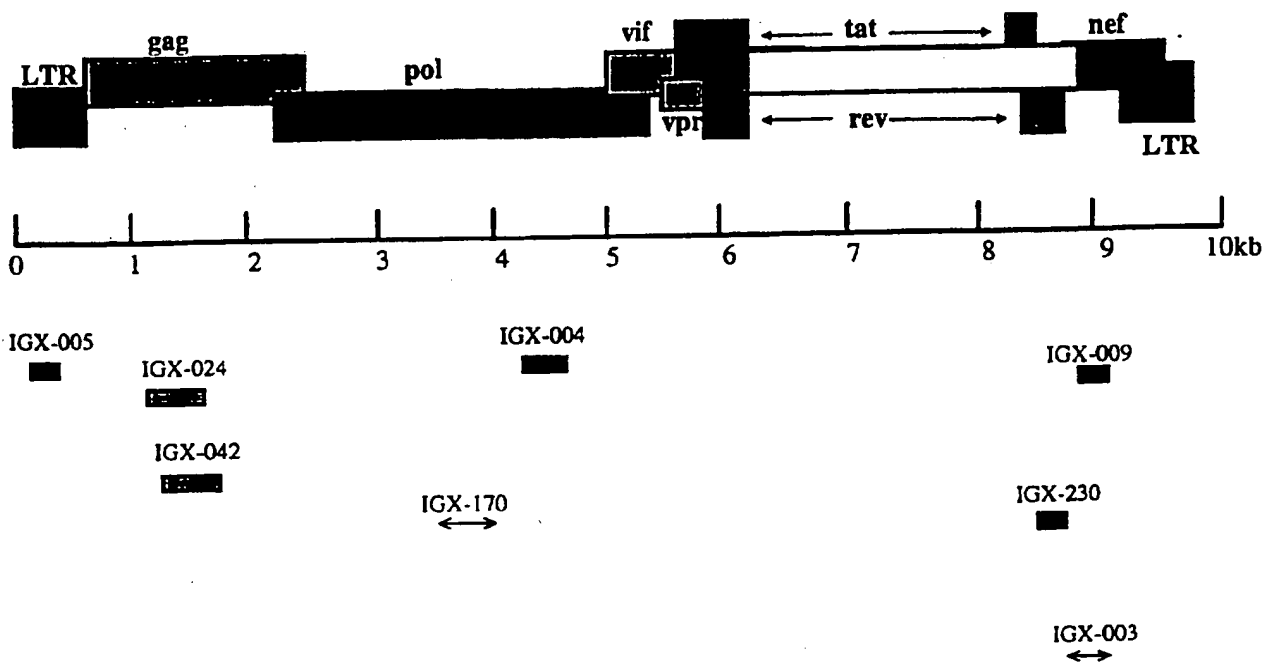CTGCGGAGCC TGTGCCTCTT CAGCTACCGC CGCT

FIG.9

TCAAATATTG GTGGAATCTC CTACAGTATT GGAGTCAGGA ACTAAAGAAT
AGTGCTGTTA GCTTGCTCAA TGCCACAGCC ATAGCAGTAG CTGAGGGGAC
AGATAGGGTT ATAGAAGTAG TACAAGGAGC TTGTAAGCTA TTCGCCACAT
ACCTAGAAGA ATAAGACAGG GCTTGGAAAG GATTTTGCTA TAAGATGGGT
GGCAAGTGGT CAAAAAGTAG TGTGGTTGGA TGGCCTACTG TAAGGGAAAG
AATGAGACGA GCTGAGCCAG CAGCAGATGG GGTGGGAGCA GCATCTCGAG
ACCTGGAAAA ACATGGAGCA ATCACAAGTA GCAATACA

FIG.10

AGCACACAAA GGAATTGGAG GAAATGAACA AGTAGATAAA TTAGTCAGTG
CTGGAATCAG GAAAGTACTA TTTTTAGATG GAATAGATAA GGCCCAAGAT
GAACATGAGA AATATCACAG TAATTGGAGA GCAATGGCTA GTGATTTTAA
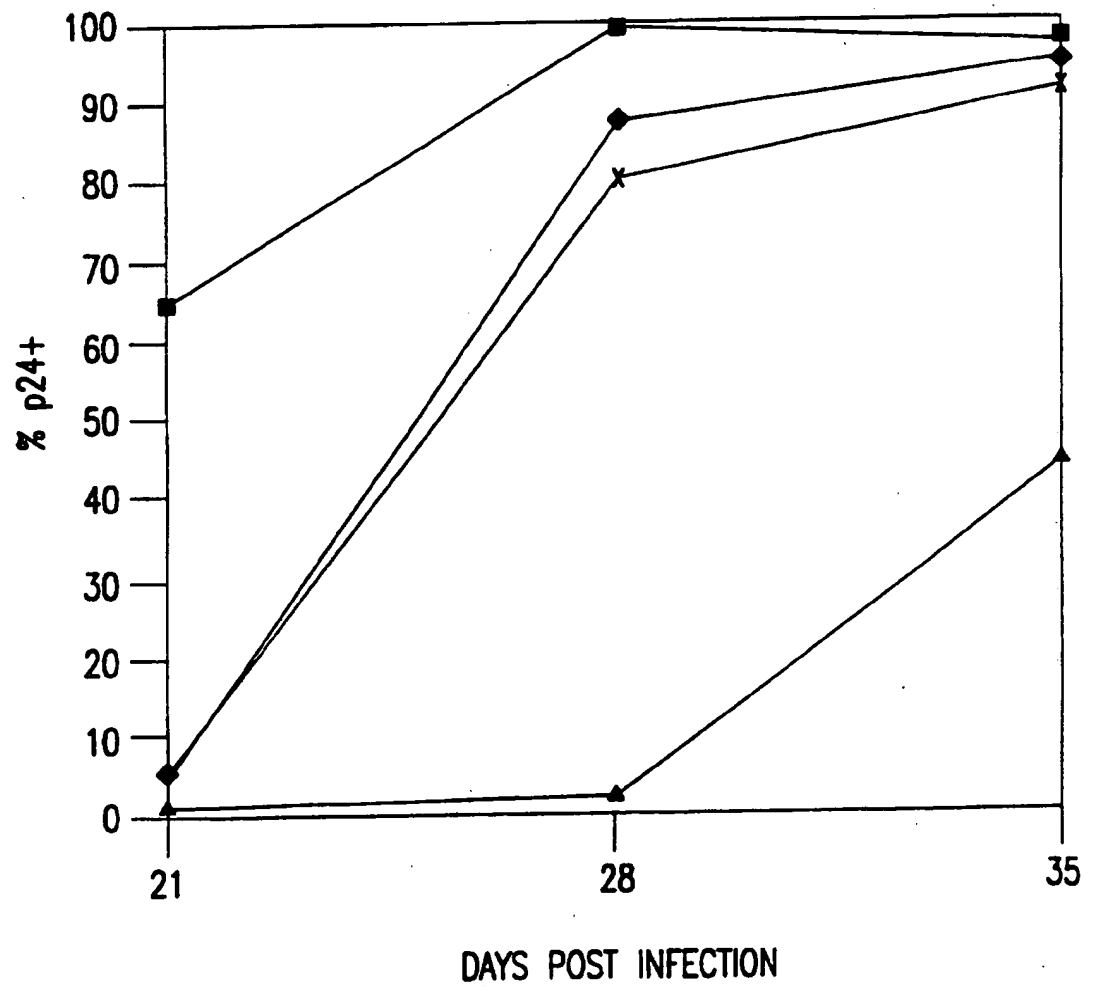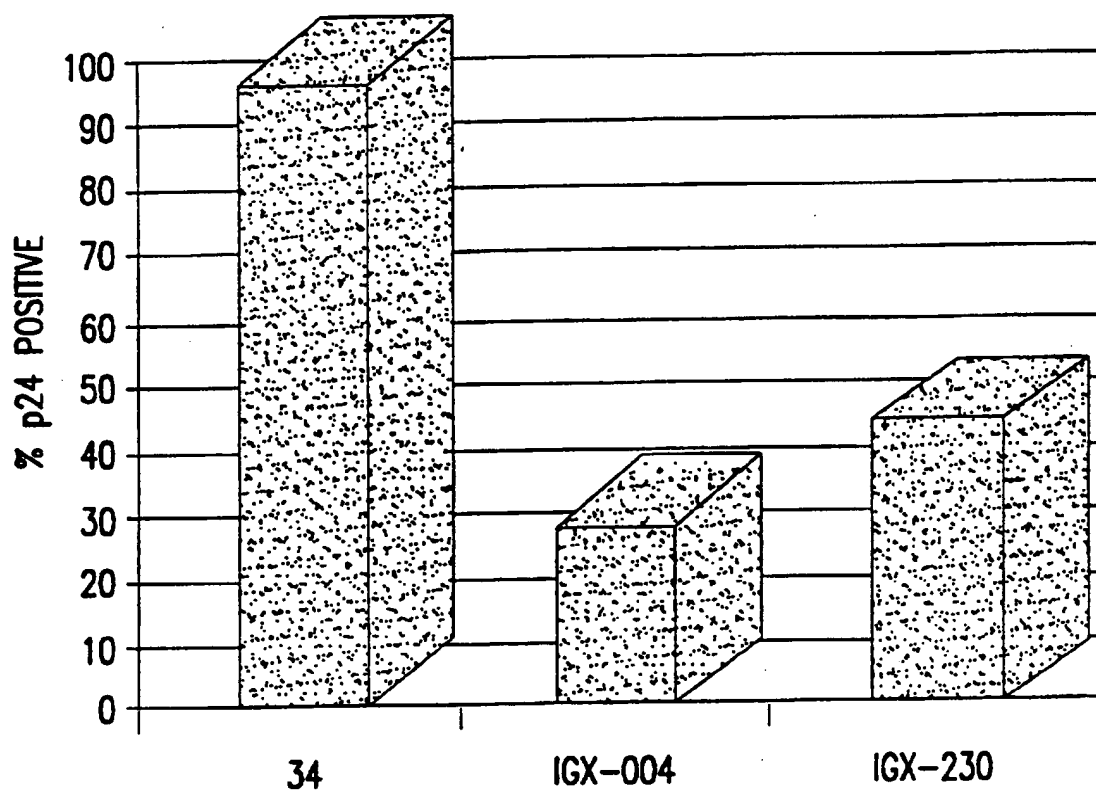CCTGCCACCT GTAGTAGCAA AAGAAATAGT AGCCAGC

FIG.11

Figure 12

FIG.13

FIG.14

FIG.15

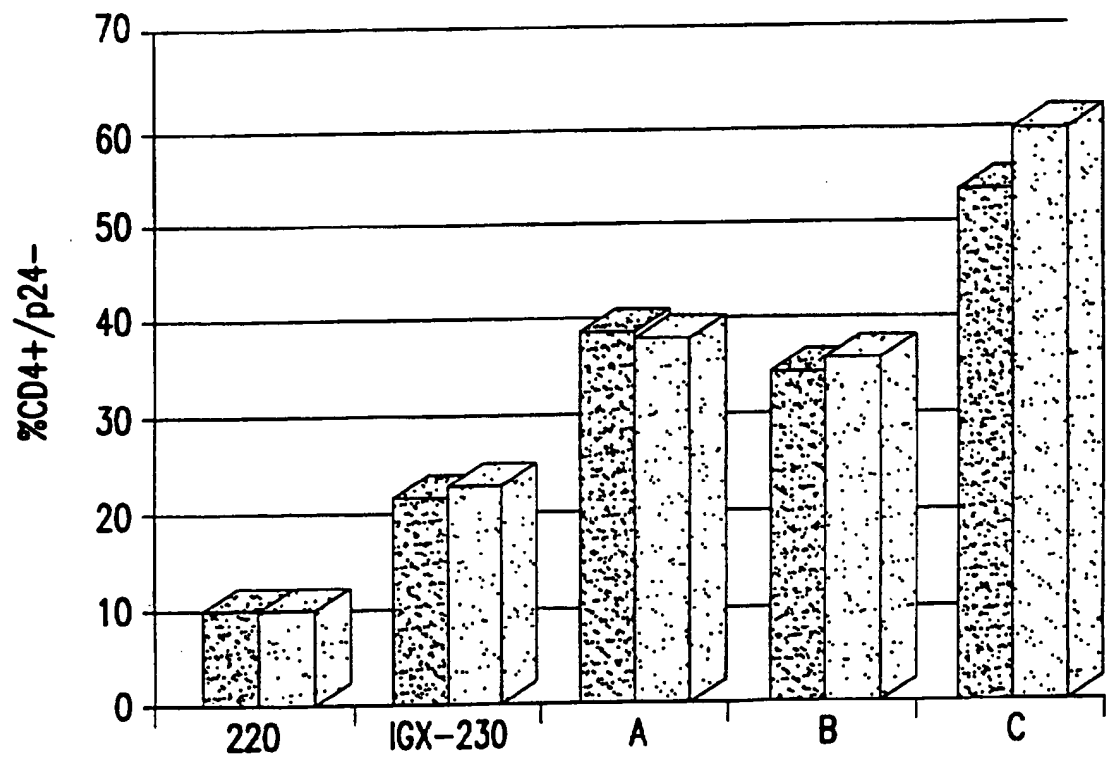DAYS POST INFECTION

FIG.16

FIG.17

DAYS POST INFECTION
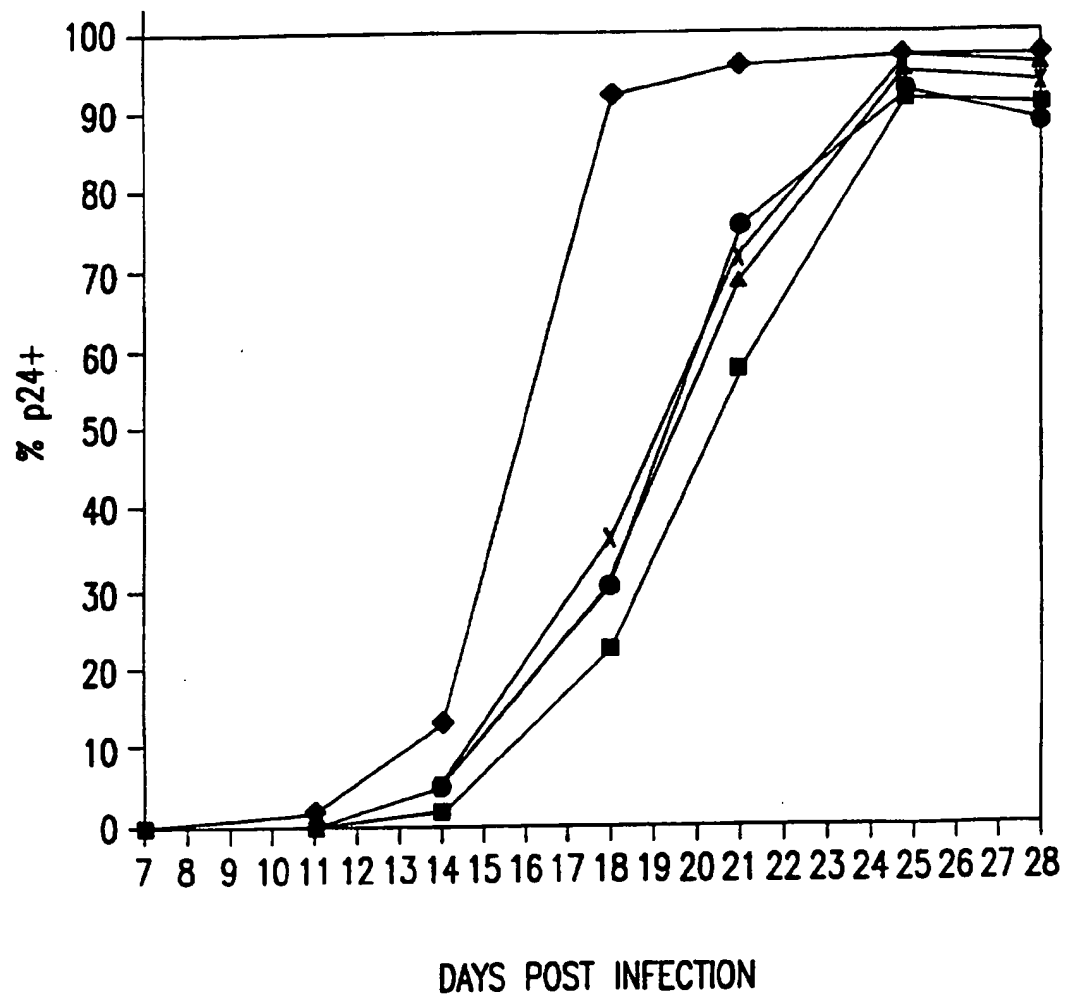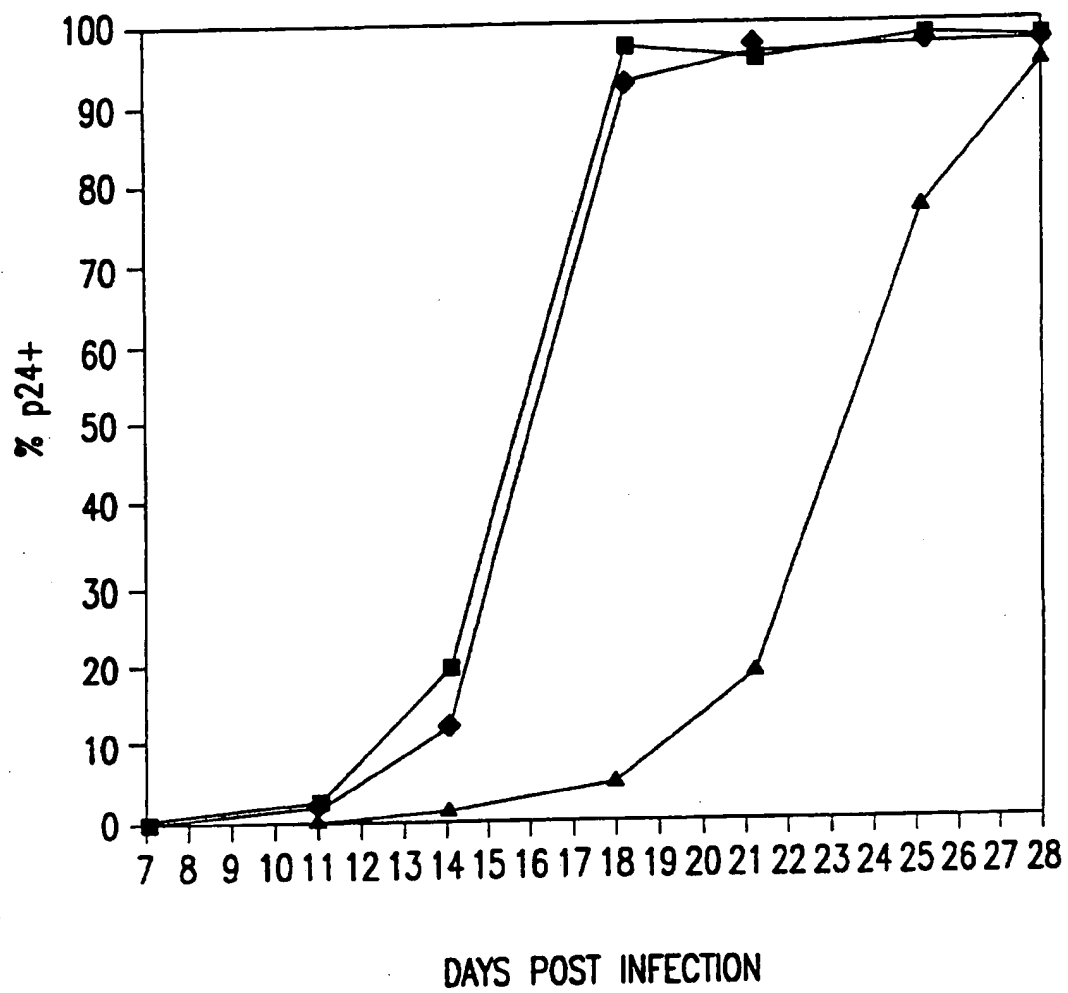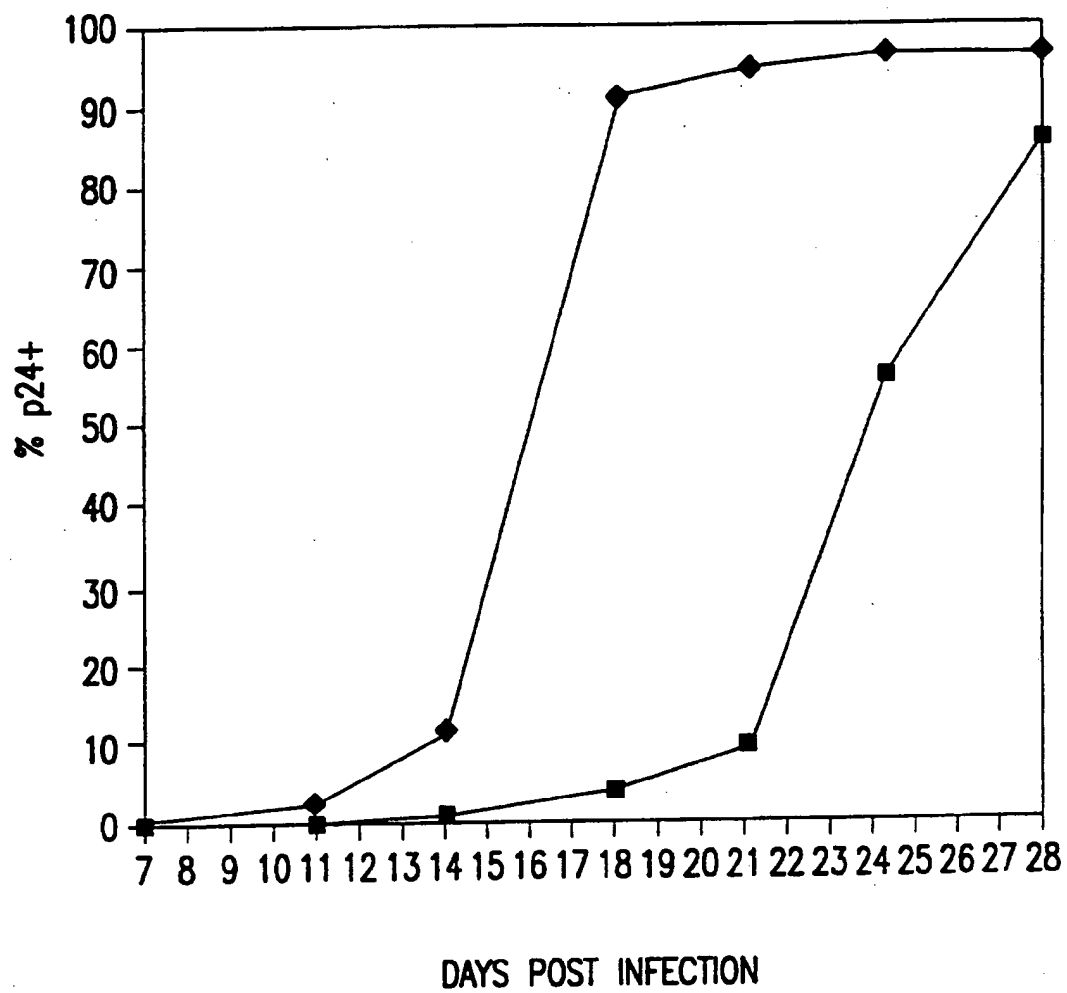
FIG.18

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/20435

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :C12Q 1/70

US CL   : 435/5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,217,889 (RONISON et al) 08 June 1993, columns 3-4, lines 45-67 and lines 35-67.	1-2,12,21,32-34,22,23
X	WO 93/11230 A1 (DYNAL AS) 10 June 1993, especially pages 7-15, 23	1,12, 21, 22, 23, 32-34
Y		2

| X | Further documents are listed in the continuation of Box C. | | See patent family annex. |

<table>
<tr><td>*</td><td>Special categories of cited documents:</td><td>"T"</td><td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td></tr>
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<tr><td>"O"</td><td>document referring to an oral disclosure, use, exhibition or other means</td><td></td><td></td></tr>
<tr><td>"P"</td><td>document published prior to the international filing date but later than the priority date claimed</td><td>"&"</td><td>document member of the same patent family</td></tr>
</table>

Date of the actual completion of the international search	Date of mailing of the international search report
11 MARCH 1997	0 4 APR 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT	PAUL GOTTLIEB

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GRAHAM, G. et al. A rapid and reliable method to create tandem arrays of short DNA sequences. Biotechniques Vol 13, No. 5, especially page 788, column 1.	2